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Laboratory Diagnosis of Amoebic Keratitis: Comparison of Four Diagnostic Methods for Different Types of Clinical Specimens▽

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Amoebic keratitis causes significant ocular morbidity in contact lens wearers. Current diagnostic methods for amoebic keratitis are insensitive and labor-intensive and have poor turnaround time. We evaluated four laboratory methods for detection of acanthamoebae in clinical specimens. Deidentified, delinked consecutive specimens from patients with suspected amoebic keratitis were assayed for acanthamoebae by direct smear analysis, culture, and PCR using two different primer sets specific for *Acanthamoeba* ribosomal DNA. The consensus reference standard was considered fulfilled when the results for any two of the four tests were positive, and the outcome measures were sensitivity and specificity. Of 107 specimens assayed over an 18-month period, 20 were positive for acanthamoebae. The sensitivity and specificity of each assay were as follows, respectively: for smear analysis, 55% (95% confidence interval [CI], 33.2 to 76.8%) and 100%; for culture, 73.7% (95% CI, 54.4 to 93.0%) and 100%; for PCR using Nelson primers, 90% (95% CI, 76.9 to 100%) and 90.8% (95% CI, 84.7 to 96.9%); and for PCR using JDP primers, 65% (95% CI, 44.1 to 85.9%) and 100%. Nelson primer PCR demonstrated a single-organism level of analytic sensitivity. The performance characteristics of the assays varied by specimen type, with contact lenses and casings showing the highest rates of detectable acanthamoebae and the highest diagnostic sensitivities for direct smear analysis, culture, and JDP primer PCR, though these results are based on small numbers and should be interpreted cautiously. These findings have important implications for clinicians collecting diagnostic specimens and for diagnostic laboratories, especially in outbreak situations.

Amoebic keratitis (AK) is a potentially blinding ocular infection caused by an *Acanthamoeba* sp. free-living protozoan parasite that is found ubiquitously throughout the environment worldwide (3). The overwhelming majority of cases of AK occur in immunocompetent contact lens wearers (14), and outbreaks have been linked to contact lens solutions contaminated with acanthamoebae or to those that fail to effectively decontaminate lenses. A recent outbreak in the United States affecting 138 people led to the recall of contact lens solutions and products by both the FDA and Health Canada and has resulted in over 150 lawsuits against the manufacturer (2, 6, 7). Plaintiffs in the lawsuits have been left with impaired vision and, in several cases, have required corneal transplants (7). Although contaminated contact lens solutions or solutions that facilitate growth are usually implicated in large outbreaks of AK, isolated cases occur in individuals who have corneal trauma or who disinfect contact lenses with tap water or other home-based preparations. Swimming and showering while wearing contact lenses are also risk factors for AK. Annual incidences of AK vary by country and are believed to be on the order of 2 to 20 cases per million contact lens wearers, accounting for 10% of the North American population (8, 12, 16, 17, 19, 21).

Clinically, AK can be easily mistaken for herpes simplex virus infection or fungal keratitis, and secondary bacterial infection is common, thus complicating diagnosis (10). Delayed diagnosis has repeatedly been associated with poor visual outcome and more-severe clinical progression (4, 5). Standard laboratory diagnostic procedures include microscopic examination of Giemsa-, periodic acid Schiff-, hematoxylin-and-eosin-, or acridine orange-stained corneal scrapings or contact lens fluids and culture of these specimens on nonnutrient agar overlaid with *Escherichia coli* or *Klebsiella pneumoniae*, and all of these procedures are limited by poor sensitivity, the requirement for technical expertise, and, in the case of culture, long turnaround time (4, 5). Due to their excellent sensitivity, molecular methods, including PCR, are increasingly being used to detect acanthamoebae in corneal specimens (9, 10, 18, 20).

As suggested by the recent outbreak and legal/medicolegal sequelae, strategies which improve upon current diagnostic methods for AK are needed (4). We herein sought to evaluate the performance of PCR for detection of acanthamoebae in clinical specimens from patients suspected of having AK in comparison to traditional methods, such as direct microscopic examination and culture.

MATERIALS AND METHODS

Samples. Consecutive specimens (corneal scrapings, contact lens solutions, and casings) from patients with suspected AK that were sent to the Central Public Health Laboratory for diagnosis between January 2007 and June 2008 were assayed using the standard diagnostic procedures (direct examination and culture) outlined below. Following completion of clinical testing, specimens were deidentified, issued unique study identifiers, aliquoted into cryovials, and stored at −20°C for future qualitative PCR testing. Per the Code of Federal Regulations, Title 45, Part 46, the use of deidentified diagnostic specimens for verifi-

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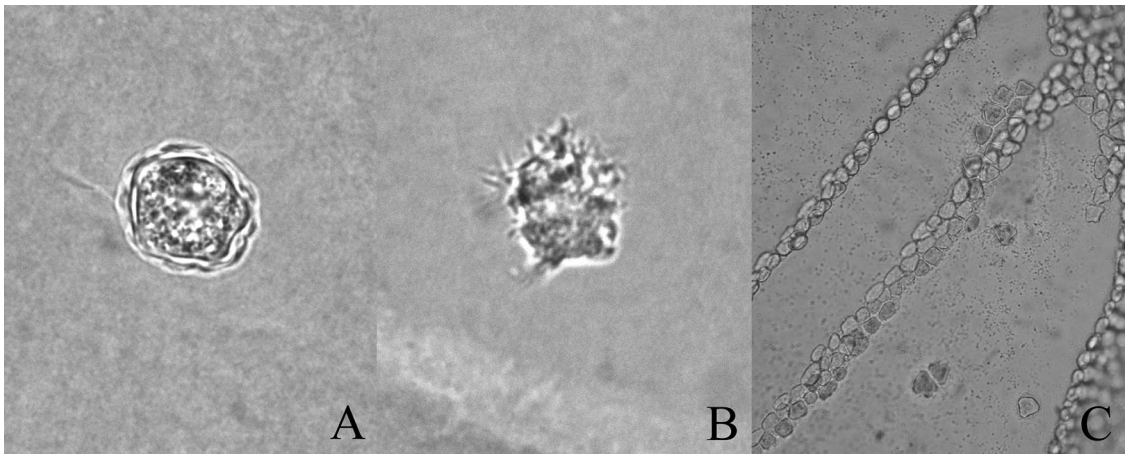

FIG. 1. (A) Cyst of the *Acanthamoeba* sp. as visualized by direct microscopy; (B) trophozoite of the *Acanthamoeba* sp. as visualized by direct microscopy; (C) positive culture of the *Acanthamoeba* sp. as visualized by inverted microscopy.

cation purposes is not considered human subject research and is therefore exempt from human subject considerations. Corneal specimens were suspended in 500 μl of sterile saline prior to storage. Contact lens solutions were stored undiluted in 500-μl aliquots. Contact lens casings were washed with 500 μl sterile saline and aliquoted as described above. The evaluators of each of the three assays were blinded to the outcomes of the other tests.

Culture. With a sterile glass pipette, 2 or 3 drops from each specimen were inoculated onto each of four culture plates: one with nutrient medium (NM) overlaid with *E. coli*, one with NM overlaid with *Klebsiella pneumoniae*, one with NM-salt overlaid with *E. coli*, and one with NM-salt overlaid with *K. pneumoniae*. Inoculated culture plates were incubated at room temperature for 8 days and observed every 2 days for growth by using an inverted microscope (Fig. 1).

Direct examination. With a sterile pipette, 2 drops of each specimen were placed onto a glass slide and allowed to air dry for 10 min. The slides were then fixed in methanol and stained with Giemsa for 8 min. After air drying, the slides were mounted with Permount and examined for cysts and trophozoites at ×200 to ×400 magnification by using a standard light microscope (Fig. 1).

Isolation of DNA from specimens. Prior to DNA extraction, frozen specimens were thawed at room temperature. In order to disrupt the integrity of *Acanthamoeba* cysts, samples were subjected to three freeze-thaw cycles in liquid nitrogen and a 56°C water bath. DNA extraction was performed using QiaAmp DNA minikits (Qiagen, Baltimore, MD).

PCR. PCR was performed in duplicate using a Qiagen *Taq* core kit (Qiagen, Baltimore, MD). The final volume of the reaction mixture was 25 μl. The PCR conditions were as follows: 94°C for 10 min, followed by 50 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 90 s, extension at 72°C for 60 s, and a final extension step at 72°C for 10 min (DNA thermal cycler; Perkin Elmer Cetus, Norwalk, CT). One primer set, specific for multicopy *Acanthamoeba* genomic sites encoding rRNA, was employed for each reaction. The first primer set had the Nelson (fwd) (5′-GTTTGAGGCAATAACAGGT-3′) and Nelson (rev) (5′-GAATTCCTCGTTGAAGAT-3′) sequences and generated a product 229 bp long (11). The second primer set had the JDP1 (fwd) (5′-GGCCCAGA TCGTTTACCGTGAA-3′) and JDP2 (rev) (5′-TCTCACAAGCTGCTAGGG AGTCA-3′) sequences and generated a product 423 to 551 bp long (18). Amplicons were visualized on 1.5% agarose minigels (Fisher Scientific, Fairlawn, NJ), stained with ethidium bromide, and observed using a UV transilluminator.

Determination of analytic sensitivity of PCR. In order to establish the lower limit of detection of acanthamoebae for the above-mentioned PCR procedure, serial dilutions of known positive samples were made. Positive-control culture plates were scraped using a scalpel, and material was transferred to a microcentrifuge tube and suspended in RPMI medium (Invitrogen Corp., Carlsbad, CA). Concentration of organisms in this initial inoculum was calculated using a hemacytometer, and an initial dilution was made using RPMI to achieve 100 organisms per μl. Serial dilutions were then made using RPMI to achieve the following concentrations: 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.19, 0.1, 0.05, 0.025, and 0.012 organisms per μl. PCR was then performed as described above, using both the Nelson and the JDP primer sets.

Confirmation of PCR products by sequence analysis. Amplicons produced by PCR with the Nelson primers were sequenced and analyzed by the Centre for Applied Genomics, Hospital for Sick Children, Toronto, Canada. Generated sequences were entered into BLAST (National Center for Biotechnology Information, NIH) for confirmation that PCR products reflected amplification of *Acanthamoeba*-specific nucleic acid.

Statistical analysis. We defined a specimen to be positive for the *Acanthamoeba* sp. when the results for any two direct microscopic examination, culture, or PCR using either primer set were positive. It was this reference standard against which each individual test was compared for sensitivity and specificity analysis. Differences in sensitivities and specificities were compared using the *z* test and are reported as percentages with 95% confidence intervals (CI). Statistical analyses were performed using SigmaStat 2.03 software (SPSS, Inc., Chicago, IL). The level of significance was set at P values of <0.05.

RESULTS

During the study period, 107 clinical specimens were examined for evidence of *Acanthamoeba* infection. Of these, 81 were corneal scrapings (76%), 17 were contact lens solutions (16%), and 5 were contact lenses (5%). The remaining specimens were one contact lens casing, one eye swab, one corneal biopsy sample, and one tear fluid sample. Of the 107 specimens evaluated, 20 (18.7%) fulfilled the reference standard criteria for a diagnosis of AK (positive results for two of four tests). Twenty-eight (26.2%) specimens were positive by one test, 10 (9.3%) were positive by three tests, and 7 (6.5%) were positive by all four diagnostic tests. Positivity rates varied among the different types of specimens, with 16% ($n = 13$) of corneal scrapings considered positive and 24% ($n = 4$) of contact lens solutions and 50% ($n = 3$) of contact lenses and casings fulfilling the criteria for a diagnosis of AK. Acanthamoebae were undetectable in eye swabs, corneal biopsy specimens, and tears.

Results for direct examination of specimens by use of a Giemsa-stained smear were positive for 11 specimens, yielding a sensitivity of 55% (95% CI, 33.2 to 76.8%) and a specificity of 100% (Table 1). Of all methods compared in this study, direct smear analysis had the poorest diagnostic sensitivity ($P = 0.006$ for comparison to culture; $P < 0.001$ for comparison to Nelson primer PCR). Diagnostic sensitivity of direct smear analysis was greatest for contact lenses and contact lens casings ($P = 0.034$ for comparison to corneal scrapings) and poorer for specimens such as contact lens solutions ($P = 0.09$

TABLE 1. Comparison of four diagnostic methods used for evaluation of 107 clinical specimens from patients suspected to have AK[a]

Assay	No. positive	No. negative	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Direct microscopy	11	96	55.0	100	100	90.6
Culture[b]	14	91	73.7	100	100	94.6
PCR						
Nelson primers	26	81	90.0	90.8	69.2	97.5
JDP primers	13	94	65.0	100	100	91.6

[a] PPV, positive predictive value; NPV, negative predictive value.
[b] Two specimens were not set up for culture.

for comparison to contact lens casings) and corneal scrapings ($P = 0.034$) (Table 2).

Results for specimen culture using four different types of NM were positive for 14 specimens, yielding a sensitivity and specificity of 73.7% (95% CI, 54.4 to 93.0%) and 100%, respectively (Table 1). Performance of culture was greatest for specimens such as contact lenses and contact lens casings, where the burden of organisms is presumably higher (Table 2). There was a trend toward poorer performance of culture for contact lens solutions than for corneal scrapings ($P = 0.078$) and contact lenses/casings ($P = 0.091$) (Table 2).

Results for amplification of *Acanthamoeba* DNA by use of PCR with the Nelson primer set were positive for 26 specimens, yielding a sensitivity of 90.0% (95% CI, 76.9 to 100.0%) and a specificity of 90.8% (95% CI, 84.7 to 96.9%) ($P = 0.004$ for comparison to culture) (Table 1). Only 18 of 26 specimens positive by Nelson primer PCR fulfilled the reference standard criteria for a diagnosis of AK, thus, eight specimens were considered to be false positive. By specimen type, Nelson primer PCR appeared to have the greatest diagnostic sensitivity for contact lens solutions, casings, and the lenses themselves, with lesser performance for specimens such as corneal scrapings, though these differences did not achieve statistical significance, possibly due to low specimen numbers (Table 2).

PCR using the JDP primer set revealed poorer diagnostic and analytic sensitivity than that using the Nelson primer set ($P < 0.001$), though the former was more specific (Table 1). The sensitivity of JDP primer PCR was 65.0% (95% CI, 44.1 to 85.9%), while the specificity was 100%. The performance of JDP primer PCR varied by specimen type, with the best diagnostic performance observed for contact lens solutions ($P < 0.001$ for comparison to corneal scrapings) and casings and actual lenses ($P = 0.034$ for comparison to corneal scrapings), with poorer performance for corneal scraping specimens (Table 2).

Serial dilutions of whole acanthamoebae were made to a concentration of <1 organism per μl and then subjected to both Nelson and JDP primer PCRs as described in Materials and Methods. PCR product was detectable at a concentration of 0.05 organisms per μl, or roughly 1 or 2 organisms per 25-μl aliquot, with the Nelson primer set, suggesting a single-organism level of analytic sensitivity. Analytic sensitivity was lower with JDP primer PCR, which detected down to 1.56 organisms per μl, or roughly 40 organisms per 25-μl aliquot.

Most organisms were not identified to the species level by sequencing, having greatest homology with the *Acanthamoeba* sp., though some shared 100% sequence similarity with *Acanthamoeba castellanii* ($n = 4$; GenBank accession numbers AY690455.1 and AF260724.1), *Acanthamoeba polyphaga* ($n = 4$; GenBank accession numbers AF132135.1 and AY026243.1), or *Acanthamoeba culbertsoni* ($n = 2$; GenBank accession number AY690459.1).

DISCUSSION

We have demonstrated that amplification of *Acanthamoeba* DNA by use of PCR with the Nelson primers (11) is a sensitive means by which to diagnose AK in a clinical laboratory setting. PCR in general had a particular performance advantage with specimens such as contact lens solutions, where a dilutional effect may be observed. While traditional direct smear analysis and culture of specimens are highly specific diagnostic methods, they are limited by high false-negativity rates, the requirement for significant technical expertise, and, in the case of culture, a very long turnaround time (4, 10, 18). The yield of culture using NM reported herein is almost identical to that reported by others using buffered charcoal yeast agar, nonnutrient agar with *E. coli*, and Trypticase soy agar with sheep or horse blood (15). Our results are also consistent with others in that culture has previously been shown to outperform JDP primer PCR in the diagnosis of AK from clinical specimens (18).

That Nelson primer PCR had a high false-positivity rate in this verification may simply reflect the outperformance of this highly sensitive molecular technique compared to that of the comparator methods. It is possible that *Acanthamoeba* DNA was detectable by the primer set at a concentration below the limit of detection of whole organisms or parasite DNA for the other assays. This represents an inherent limitation of any

TABLE 2. Performance characteristics of four methods for diagnosis of AK used for evaluation of 107 clinical specimens by specimen type

Assay	Value (%) for specimen type					
	Corneal scraping ($n = 81$)		Contact lens solution ($n = 17$)		Contact lenses and casings ($n = 6$)	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Direct microscopy	46.2	100.0	50.0	100.0	100.0	100.0
Culture[a]	75.0	100.0	50.0	100.0	100.0	100.0
PCR						
Nelson primers	84.6	91.2	100.0	92.3	100.0	66.7
JDP primers	46.2	100.0	100.0	100.0	100.0	100.0

[a] Two specimens were not set up for culture.

diagnostic evaluation in the absence of a well-performing reference standard (1).

Corneal specimens for the diagnosis of AK are notoriously difficult to obtain, and few patients tolerate corneal scraping well (5, 10). Obtaining a sufficient volume of clinical specimen to facilitate decent smear or culture yields is challenging (5, 10). Similarly, large-volume specimens such as contact lens solutions have a demonstrable dilutional effect and are therefore subject to poor smear and culture results, as demonstrated by our specimen-based performance analysis. Thus, diagnostic methods that detect very few organisms in a clinical specimen are clearly advantageous. We have demonstrated an analytic sensitivity for Nelson primer PCR to the single-organism level. Such analytic sensitivity has implications not only for routine diagnosis of AK but also for a test of cure, where one would expect the burden of acanthamoebae to be extremely low in clinical specimens. In addition, Nelson primer PCR could prove to be a rapid, sensitive tool for screening batches of contact lens solutions in outbreak situations.

In the clinical laboratory setting, care must be taken to balance maximization of *Acanthamoeba* culture yield through prolonged incubation and production of a timely and clinically useful result. In the case of AK, prompt initiation of appropriate therapy is necessary to limit ocular morbidity and optimize visual outcome (4, 5). Thus, employment of a rapid, sensitive screening tool, such as Nelson primer PCR, followed by a rapid, specific confirmatory test, such as JDP primer PCR, may offer benefits beyond those achieved through direct specimen microscopy and culture alone. Strategies which simplify the procedure for laboratory investigation of AK are likely worthwhile and cost-effective (4).

Of particular interest to clinicians is that the kind of specimen most frequently submitted to the laboratory, the corneal scraping, statistically had the lowest rates of detectable whole organisms and *Acanthamoeba* DNA by all tests but Nelson primer PCR. While this may simply reflect that patients with actual AK in our sample were more likely to be contact lens wearers and thus have contact lens-related specimens to submit, it may also reflect that the burden of organisms in contact lenses, cases, and fluids is greater than what is seen in a corneal scraping. This hypothesis is supported by our specimen-based performance analysis of the individual diagnostic assays, though these results should be interpreted cautiously given the low numbers of positive samples by each specimen type evaluated. While detection of acanthamoebae from contact lenses, fluids, or casings does not strictly confirm the diagnosis, it is virtually diagnostic in the setting of a compatible clinical history (10, 13). Thus, submission and processing of these types of atypical specimens may be as important as those for corneal scrapings. Other potential explanations for the noted assay performance disparities by specimen type involve the presence of PCR inhibitors in the corneal tissue itself and the low volume of clinical material obtained by corneal scrapings. Future evaluation of the disparity in yield by specimen type where the potential bias of contact lens use can be controlled is warranted.

The evaluation herein highlights the limitations of commonly employed diagnostic assays for AK and supports the potential utility of at least one primer set for molecular detection of acanthamoebae in clinical specimens. PCR had a clear diagnostic advantage over conventional techniques for large-volume specimens, such as contact lens solutions, where a dilutional effect would be expected. PCR is less labor-intensive than culture, requires fewer specialized technical skills, is more sensitive, and offers a much more rapid turnaround time, all of which culminate in the ability of the clinical laboratory to produce a meaningful, clinically relevant result. We would encourage clinicians to consider submission to the laboratory of contact lens-associated materials in addition to corneal scrapings from any patient in whom AK is suspected.

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D.S.M. contributed to data collection and interpretation, was responsible for enrolling specimens, and, along with T.Y.L., conducted the molecular analyses. B.Y. and D.E.L. contributed to study design, implementation, and data interpretation. A.K.B. contributed to study design, data collection, analysis, and interpretation and was primarily responsible for writing the manuscript. All authors contributed to and critically appraised the manuscript.

We have no conflicts of interest to declare.

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